Gut bacterial DNA translocation is an independent risk factor of flare at short term in patients with crohn's disease

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Abstract: OBJECTIVES We aimed at evaluating bacterial DNA (bactDNA) presence in blood of Crohn's disease (CD) patients in remission as an independent risk factor of flare at 6 months. METHODS This is a prospective, multicenter study on CD patients with Crohn's disease activity index (CDAI)<150. The primary end point was time-to-relapse as evaluated by CDAI>150 in the following 6 months. BactDNA in blood, the nucleotide-binding oligomerization domain containing 2 (NOD2) genotype, and serum cytokine levels were determined at baseline. RESULTS A total of 288 patients were included. BactDNA was detected in 98 patients (34.0%). A variant-NOD2 genotype was identified in 114 patients (39.6%). Forty patients (14%) relapsed during follow-up. Multivariate survival analysis identified bactDNA as an independent risk factor of flare (hazard ratio (HR) 8.75 (4.02-19.06) 95% confidence interval (CI)). Hospitalization, surgery, switch of treatment, initiation and escalation of anti-tumor necrosis factor (TNF) therapy, steroids initiation, and increased fecal calprotectin levels at 6 months were associated with bactDNA at baseline. A logistic regression analysis showed bactDNA as an independent and significant predictive factor of hospitalization (odds ratio (OR) 11.9 (3.4-42.3); P<0.001), steroids startup (OR 8.5 (2.7-27.1); P<0.001), and switch of treatment (OR 3.5 (1.6-7.7); P=0.002) at 6 months. No relationship was observed between bactDNA and mucosal lesions in patients with colonoscopy at admission. Serum pro-inflammatory cytokines were significantly increased in patients with bactDNA or a variant-NOD2 genotype. The combination of both factors induced decreased anti-TNF- levels and a higher percentage of patients on intensified anti-TNF therapy. CONCLUSIONS BactDNA is an independent risk factor of relapse at 6 months in CD patients. BactDNA is also independently associated with an increased risk of hospitalization, switch of treatment, and steroids initiation.

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Title:

Gut bacterial DNA translocation is an independent risk factor of flare at short-term in patients with Crohn's Disease.

Short title:

Crohn's disease and bacterial DNA

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Abbreviations: CD: Crohn's disease; bactDNA: bacterial DNA; NOD2: nucleotide-binding oligomerization domain containing 2. TNF-α: tumor necrosis factor alpha.

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Authors' contribution

AG, LS, MG, RL, AM: patients' inclusion, treatment and follow-up

PZ, AG, OJ, MS, JMG, RF: experimental data, acquisition and analysis

JS, RW, GR: critical revision and clinical considerations

RF: study concept and design, manuscript writing

Abstract

Background: Translocation of bacterial DNA (bactDNA) is a frequent event in patients with Crohn's disease. We aimed at evaluating bactDNA presence in blood of CD patients in remission as an independent risk factor of flare at 6 months.

Methods: Prospective, multicenter study on CD patients with CDAI<150. The primary endpoint was time-to-relapse as evaluated by CDAI>150 in the following 6 months. BactDNA in blood, the *NOD2* genotype and serum cytokine levels were determined at baseline.

Results: 288 patients were included. BactDNA was detected in 98 patients (34.0%). A variant-NOD2 genotype was identified in 114 patients (39.6%). Forty patients (14%) relapsed during follow-up. Multivariate survival analysis identified bactDNA as independent risk factor of flare (HR 8.75 [4.02-19.06] 95%CI). Hospitalization, surgery, switch of treatment, initiation and escalation of anti-TNF therapy, steroids initiation and increased fecal calprotectin levels at 6 months were associated with bactDNA at baseline. A logistic regression analysis showed bactDNA as an independent and significant predictive factor of hospitalization (OR 11.9 [3.4-42.3]; p<0.001), steroids startup (OR 8.5 [2.7-27.1]; p<0.001) and switch of treatment (OR 3.5 [1.6-7.7]; p=0.002) at 6 months. No relationship was observed between bactDNA and mucosal lesions in patients with colonoscopy at admission. Serum proinflammatory cytokines were signifcantly increased in patients with bactDNA or a variant-NOD2 genotype. The combination of both factors induced decreased anti-TNF-alpha levels and a higher percentage of patients on intensified anti-TNF therapy.

Conclusion: BactDNA is an independent risk factor of relapse at 6 months in CD patients. BactDNA is also independently associated with an increased risk of hospitalization, switch of treatment and steroids initiation.

Keywords:
Crohn's disease, bacterial DNA, NOD2, relapse, anti-TNF.

Introduction

The translocation of bacterial genomic fragments (bactDNA) into blood is a frequent event arising in up to 40% of patients with Crohn's disease [1, 2]. The multifactorial etiology of the disease includes partial genetic susceptibility and immunological interactions between the host and commensal bacteria [3-6]. An impaired host-microbe immune relationship, probably driven by allelic variants in these predisposition-associated genes, has been associated with the development of inflammatory bowel disease [7] and may contribute for the translocation of bacterial antigenic products, which in turn, are closely related to a sustained inflammatory response in CD patients [8].

BactDNA is a potent immunogenic bacterial product able to trigger a Th1-biased pro-inflammatory response through toll-like receptor (TLR)-9 recognition and nuclear factor kappa-B (NFk-B) activation [9-12]. Pro-inflammatory mediators such as Tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-12 and Interferon gamma (IFN-γ) are significantly increased in response to bactDNA [13-16] and are also increased in active CD patients [17-20]. On the other hand, we have previously shown that CD patients bearing a variant *NOD2* genotype have significantly decreased phagocytic and bactericidal activities, as well as increased serum TNF-α levels in response to bactDNA [8]. Variants in this gene have been associated with predisposition to CD [21-24], the regulation of the gut immune response and microbiota [25, 26], and even disease complications [27, 28].

Controlling the adverse effects of an exacerbated pro-inflammatory reaction in CD patients is of great relevance for the course of disease. Continued flares due to failure of medical treatment, bowel obstruction, fistulae or abscess formation is associated, besides pain and discomfort in patients, with need for hospitalization and, eventually, surgery both in luminal and perianal disease [29]. Current therapies are aimed at preventing/reducing the inflammatory outbreaks and to maintain clinical remission in CD patients by targeting some of the cytokines mentioned above. BactDNA translocation, which deeply influences the cytokine network, may constitute a disrupting factor that imbalances therapeutic-induced homeostasis in these patients, increasing their risk for relapse.

Thus, the aim of the present investigation has been to determine whether bactDNA presence in blood of CD patients in remission constitutes an independent risk factor of relapse and other complications in the short-term, controlling the results by the NOD2 gene status.

Patients and methods

Patients and study design.

Consecutive patients diagnosed with Crohn's disease and controlled at three hospitals in the area of Alicante, Spain, who were in remission, as determined by CDAI<150 were included in this prospective, observational multicenter study. The diagnosis of CD was established according to standard clinical, endoscopic, histological and radiographical criteria [30]. Patients treated with antibiotics in the previous 4 weeks, patients with signs of active infection and those who refuse to sign informed consent to participate in the study were excluded. Twenty-five healthy controls were included in the study of NOD2 allelic variants distribution. The Ethics Committee of the each hospital approved the study protocol.

Usual clinical and analytical variables in the management of CD patients were recorded. All patients were Caucasian of Mediterranean ethnicity and were classified according to the Montreal classification [31]. All included patients received diaries to record symptoms 1 week prior to inclusion and sample collection. Therapies were grouped in four categories: No immunosuppressors (IS)/no anti-TNF, which included patients without any treatment and those on mesalazine; IS (patients on azathioprine or methotrexate), anti-TNF (patients on infliximab or adalimumab), and the combination of IS with anti-TNF. Anti-TNF intensified therapy was defined either by an increased dose or an increase in the frequency of infusions versus dosing or schedule upon start of treatment.

Blood samples were obtained for routine haematological and biochemical studies at inclusion and inoculated in aerobic and anaerobic blood culture bottles, 10 ml each. Simultaneously, two separate blood samples were inoculated under aseptic conditions in rubber-sealed sterile Vacutainer SST II and K3E tubes, respectively (BD Diagnostics, Erembodegem, Belgium) that were never exposed to free air.

Patients were followed up for 6 months. The study's primary endpoint was time to relapse as evaluated by CDAI>150, or by clinical symptoms and endoscopic findings or fecal calprotectin>250ug/g in patients with previous surgery. Secondary endpoints in

the follow-up timeframe were the incidence of complications such as hospitalizations, switch of treatment, anti-TNF intensification, surgery or initiation of steroids.

Identification of bactDNA fragments and NOD2 genotyping.

Genomic DNA was isolated from $5x10^6$ cells with the QIAmp DNA Blood Minikit (Qiagen, Hilden, Germany). BactDNA was identified by running a broad-range PCR with 5'-AGAGTTTGATCATGGCTCAG-3' as forward and 5'-ACCGCGACTGCTGCTGGCAC-3' as reverse primers, followed by partial nucleotide sequencing of a conserved region of 16SrRNA gene. The tree common NOD2/CARD15 allelic variants at SNP-8 (R702W, rs2066844), SNP-12 (G908R, rs2066845) and SNP-13 (L1007finsC, rs2066847) were genotyped by TaqMan technology (Applied Biosystems) using commercially available TaqMan SNP Genotyping Assays and TaqMan Genotyping Master Mix on a 7900HT Fast Real-Time PCR System using SDS 2.2 Software (Applied Biosystems), as previously described [8]. A variant NOD2 genotype was defined as carrying any of the three studied variants either in homozygosis or heterozygosis. To minimize error, all genotyping results were scored twice and the second assessor was not aware either of each patient disease-status or the first genotype results. No missing genotypes were present.

Serum cytokine and free anti-TNF-α levels. Presence of anti-drug antibodies.

Serum TNF-α, IFN-γ, IL-12p40 levels were determined by flow cytometry using Cytometric Bead Arrays (CBA) in a FACs Canto II (Becton Dickinson, San Jose, CA). Enzyme-linked immunosorbent assays (ELISAs) were carried out to measure free infliximab and adalimumab levels and to detect anti-drug antibodies (Matriks Biotek, Ankara, Turkey) according to the manufacturers' instructions. All samples were tested in triplicate and read in a Sunrise Microplate Reader (Tecan, Männedorf, Switzerland). The detection limit for each cytokine assay varied between 2–5 pg/mL and between 10-30ng/mL in the case of free anti-TNF-α kits. Standard curves were generated for every plate and the average zero standard optical densities were subtracted from the rest of the standards and samples to obtain a corrected concentration for all parameters. The presence of anti-drug antibodies was evaluated by a cut-off value estimated by multiplying the optical density (OD) of the zero standard by 3, as indicated by the manufacturers. Samples were considered positive when the ratio sample OD/zero standard OD was higher than 3.

Statistical analysis.

Continuous variables were reported as mean ± standard deviation or 95% confidence interval, and categorical variables were expressed as frequencies and percentages. In a first univariate analysis, differences between patient groups were analyzed using the U-Mann Whitney test for quantitative data and Chi-square test for qualitative data. Time to relapse was analyzed using a Kaplan-Meier survival approach and log-rank test according to bactDNA and group of therapy (NOD2/CARD15 mutations status). A multivariate Cox proportional regression analyses to study factors significantly related to time to relapse was performed in two steps: first, an univariate analyses using bactDNA, NOD2 genotype, groups of therapy and clinical and demographic variables as independent variables and then variables showing a p value lower than 0.1 were considered in a multivariate analysis. The associations between clinical and demographic data and secondary outcomes with at least 10 events each as hospitalization, switch of treatment and the startup of steroids during the study follow-up were evaluated using a multiple logistic regression analysis when time to event was not available. Statistical significance was considered at p values less than 0.05. Statistical analysis was performed using SPSS v15 and R software.

Results

Characteristics of patients.

A total series of 288 consecutive patients fulfilling inclusion and exclusion criteria were included from 2012 to 2014. All patients completed the 6-month follow-up period. Clinical and analytical characteristics of the study cohort are shown in Table 1. Briefly, mean age was 42±15 years and 53% were male. Mean CDAI was 62.6±33.1 and mean fecal calprotectin was 79.5±91.7 μg/g. Forty percent of the study population were active smokers and 25% had a history of previous surgery. Ileal disease was present in 45% of patients whereas colonic or ileo-colonic disease was present in 50% of patients. Twenty-one percent of patients presented with perianal disease. Regarding therapies at inclusion, 28% of patients were on anti-TNF, either alone or combined, and 55% were receiving immunosuppressors.

Bacterial DNA translocation and NOD2 allelic variant distribution in CD patients

BactDNA was detected in blood samples from 98 CD patients in remission (34.0%). Partial sequencing analysis identified 23 species that are resumed in Table 2. As can be observed, 77.6% of identified bactDNA belonged to the *Enterobacteriaceae* family and *E. coli* was the mainly identified species among bactDNA-positive CD patients (38.7%). BactDNA from gram-positive microorganisms was detected in 22.4% of CD patients with bacterial translocation. The amount of amplified bactDNA was not significantly different between species, family or gram distribution.

The distribution of patients according to the presence of bactDNA in blood can be followed in Table 1. No significant baseline clinical or analytical differences were observed between patients according to bactDNA translocation, including the groups of therapy.

A variant NOD2 genotype was identified in 114 patients (39.6%). Distribution of genotypes and allelic frequencies in CD patients is shown in Supplementary Table 1. All variants were found to be in the Hardy–Weinberg equilibrium in the controls. BactDNA was present in 37 patients with a wild type NOD2 genotype and in 61 patients with a variant NOD2 genotype (32.5% vs 35%, respectively; p=ns). There were no significant

differences either in any of the studied NOD2 variant allelic frequencies according to bactDNA patients' distribution.

Evaluation of relapse during the following 6 months according to the presence of bactDNA.

Forty CD patients (14%) out of the overall series of patients included relapsed during the study follow-up period. Thirty-two patients out of 98 with bactDNA fragments in blood (33%) *vs* 8 patients out of 190 without bactDNA (4%, p=0.001) presented a flare-up. Relapse during follow-up was also significantly different according to the group of therapy (no IS/no anti-TNF 14.4%; IS 12.4%; anti-TNF 18%; IS + anti-TNF 32.3%, p=0.004). No other baseline characteristics of patients were different when considering relapse at 6 months. Interestingly, 16 wild-type *NOD2* (14%) and 24 variant *NOD2* (13.8%) genotyped patients relapsed during the study follow-up, without significant differences in the frequency of studied allelic variants.

The survival analysis of time to first relapse according to bactDNA presence in blood and to the group of therapy is shown in Figure 1. In the Cox proportional hazards multiple regression analysis, both variables remained independently significant, as can be followed in Table 3. There was a significant difference in the relapse rate between patients on IS combined with anti-TNF compared with patients on no IS/no anti-TNF (p=0.002).

Clinical evolution of patients

Nineteen patients (6.7%) of the overall series of patients included required hospitalization during the study follow-up. The number of patients who needed hospitalization was significantly increased in the presence of bactDNA at baseline (16.3% *vs* 1.6 % in patients without bactDNA at baseline, p<0.001). Five patients with bactDNA (5.1%) required surgery in the following six months (bowel resection in 3 patients and drainage of perianal abscess and fistulae in 2 patients) whereas no surgery was required in this time-frame in the group of patients without bactDNA at baseline. Other significant differences in the 6-month clinical evolution between patients with and without bactDNA at baseline were the switch of treatment, the initiation and the escalation of anti-TNF therapy and the initiation of steroids (Table 4A).

Among analytical parameters at 6 months, only fecal calprotectin was significantly increased in CD patients with bactDNA at baseline compared with those without evidences of this bacterial antigen in blood (60.06±98.5 *vs* 151.31±188.98 ug/g; p=0.001) (Table 4A).

Hospitalization, the switch of treatment, the escalation of anti-TNF therapy and the startup of steroids were also significantly different between patients compared by the group of therapy (Table 4B). Any of the analytical parameters evaluated at 6 months other than fecal calprotectin was significantly different between CD patients distributed by group of therapy (Table 4B).

A logistic regression analysis was performed controlling by all baseline clinical and analytical variables and showed bactDNA as the only significant predictive factor of hospitalization (OR 11.9 [3.4-42.3]; p<0.001) and the startup of steroids (OR 8.5 [2.7-27.1]; p<0.001) at six months. BactDNA (OR 3.5 [1.6-7.7]; p=0.002) and active smoking habit (OR 2.7 [1.2-6.4]; p=0.02) were both significant predictive factors of switch of treatment.

Gut bactDNA translocation does not correlate with endoscopic findings of mucosal lesion.

Colonoscopies in the previous month to admission were available from 109 out of 288 CD patients (38%) included in the study. Of those, 54 patients (49.5%) presented evidences of mucosal lesions. Table 5 details their distribution in patients according to bactDNA presence in blood, NOD2 genotype and group of therapy. As can be observed, no significant differences were found between patients in any case. Of interest, no relationship could be established between mucosal lesions and the translocation of bactDNA fragments into blood. In the Cox proportional hazards multiple regression analysis, bactDNA (HR 7.24 [2.33-22.51]; p=0.001) and the group of therapy (HR 7.06 [1.42-35.03]; p=0.017) remained independently significant, Endoscopic evidences at baseline of mucosal lesions were associated with higher relapse rates than evidences of no lesions, although this difference in the survival analysis was not statistically significant (HR 1.72 [0.62-4.73]; p=0.282) (Supplementary Figure 1). The rest of complications studied was not statistically different, either.

The probability of relapse in bactDNA-positive was higher in patients with endoscopic evidence of mucosal lesions compared with no lesions (8/15 [53%] *vs* 4/18 [22%]; p=0.08). This difference did not reach statistical significance probably due to reduced sample size of the resultant subgroups.

None of the analytical parameters evaluated at 6 months were statistically different according to patients' distribution by endoscopic evidence of mucosal lesions (data not shown).

Gut bactDNA translocation regulates serum cytokine and free anti-TNF levels in CD patients.

Serum levels of pro-inflammatory cytokines were measured at baseline in all included patients. The presence of bactDNA in blood and a variant *NOD2* genotype significantly increased TNF-alpha compared with levels in patients without bactDNA (Figure 2A) or with a wild-type *NOD2* genotype (Figure 2B), respectively. The distribution of patients by group of therapy did not reveal any significant differences in serum TNF-alpha levels (Figure 2C).

Serum TNF-alpha levels were independently evaluated in patients on anti-TNF therapy, either alone or combined with IS, together with free anti-TNF levels. The distribution of patients by the presence of bactDNA and the NOD2 genotype revealed that the highest TNF-alpha and the lowest anti-TNF-alpha levels were present in patients with bactDNA and a variant *NOD2* genotype (Figure 2D and 2E). Of interest, five patients showed anti-drug antibodies in blood, 2 on anti-TNF monotherapy and 3 on anti-TNF and IS combined therapy, and were not included in these figures. Eighteen patients out of 81 (22.2%) were on intensified anti-TNF therapy. Need for intensification was clearly related with the presence of bactDNA (15 out of 18 patients, 83.3%). A variant NOD2 genotype was present in 2 intensified patients without bactDNA (4.2%) and12 intensified patients with bactDNA (35.3%, p=0.01). Figure 2F shows the percentage of patients on intensified anti-TNF therapy in each subgroup of patients. A negative correlation was established between TNF-alpha and free anti-TNF-alpha in blood of the overall series of CD patients on anti-TNF therapy ($r=-0.57$; $p<0.001$).

The serum levels of IL-6, IFN-gamma and IL-12 in patients either distributed by bactDNA, *NOD2* genotype or group of therapy are detailed in Supplementary Table 2. Also, no differences in serum levels of any of the pro-inflammatory cytokines were observed in patients distributed by endoscopic findings of mucosal lesion or healing (data not shown).

Discussion

This prospective, observational study was designed to evaluate the clinical implications in the short-term of circulating bactDNA fragments in blood of CD patients in remission. Results show that CD patients with bactDNA are at significantly increased risk of relapse at 6 months. Other clinical complications such as the need for hospitalization, switch of treatment, startup of steroids or surgery are also significantly increased in the group of CD patients with bactDNA fragments in blood compared with those showing no evidence of this bacterial antigen type in the circulation. These results show the relevance of identifying circulating bacterial antigens in CD patients and may provide the rationale for a distinct therapeutic management to maintain remission in the subgroup of bactDNA-positive CD patients.

Different factors have been associated with a disabling course of CD in the following years, as disease behavior, the age of onset and the initial requirement of steroids [32]. The chronic inflammatory condition that characterizes CD is recurrently altered by disease flare-ups and, eventually, irreversible lesions that may result in resection surgery. Some of the key elements that have been associated with disease exacerbation are the use of non-steroidal anti-inflammatory drugs (NSAIDs) [33], an active smoking habit [34], and non-compliance with medication [35, 36]. Even the emotional stress has been suggested as a trigger of flare by CD patients [37, 38]. The etiology of CD includes, among other factors, the inadequate interaction between the host and intestinal microorganisms [3, 5, 6], as well as a partial genetic predisposition mainly derived, but not exclusively, from allelic variants of the *NOD2* gene [21, 39, 40]. However, no studies were available regarding the evaluation of either bacterial translocating antigens or variant *NOD2* genotypes on the risk of disease relapse in the short-term in CD patients.

We have described in the past the translocation of bactDNA into blood of CD patients, and also the partial genetic influence of variant NOD2 genotypes on an impaired immune response in CD patients [1, 8, 41]. In this study, we evaluated the effect of bactDNA translocation and NOD2 genotype on relapse at six month in CD patients in remission. As can be observed in Table 3, bactDNA translocation constituted a significant independent risk factor of flare in the multivariate survival analysis, whereas the variant

NOD2 genotype did not. BactDNA had been reported to contribute to perpetuation of sustained intestinal inflammation [4], which may favor relapse in CD patients. This fact could account for the association observed herein between bactDNA and the increased risk of flare. Along with bactDNA translocation, the distribution of CD patients by group of therapy was also shown as an independent risk factor of relapse at six months. Patients on combined therapy with IS and anti-TNF were at higher risk than patients without treatment or those with mesalazine. This is an expected result, as patients on heavier therapies are more likely identifying more aggressive disease phenotypes, which obviously account for relapse.

The endoscopic evidence of mucosal lesion was evaluated as predictive factor of relapse at 6 months in the subgroup of patients with available colonoscopy in the previous month to admission. The presence of mucosal lesions almost doubled the risk of relapse but this increment was not statistically significant, probably as a consequence of an unpowered sample size. However, in this same subgroup of patients, bactDNA presence and the group of therapy remained as independent and significant risk factors of flare at six months. Interestingly, the relapse rate among bactDNA-positive patients was more frequent in those with mucosal lessions (53% *vs* 22%), suggesting that, despite no lesion is needed for bactDNA to translocate, the presence of mucosal aphtous lesions or ulcers may sinergistically act with bactDNA as inflammatory triggers conducting to relapse. On the other hand, although mucosal healing has been associated with reduced hospitalization [42] and surgery rates [43] or maintenance of clinical remission [44], we do not find differences in these complications either compared with patients showing mucosal lesions. The shorter follow-up period compared with those studies may account for the discrepancies.

Besides risk of flare at six months, the presence of bactDNA in blood of CD patients in remission was also significantly associated with an increased risk of hospitalization, surgery, switch of treatment, anti-TNF intensification, startup of steroids and increased fecal calprotectin levels in this time-frame (Table 4A). These results clearly identify bactDNA as a marker of relevant disease complications that not only affects patients' welfare but also could have an economic impact on the health system.

From an immunologic point of view, bacterial elements such as DNA are known to induce an increased inflammation through activation of the NF-KB signaling pathway after host recognition by specific receptors in different immune cell types [9, 16]. The gut epithelial barrier plays a crucial role in regulating the uptake of luminal antigens. The episodic translocation of bactDNA may be suggesting either a dysfunction in the epithelial cell layer or an intestinal bacterial overgrowth. In fact, inflammatory bowel diseases have been associated with an altered gut microbiota composition [45]. Anyhow, results described herein showing the presence of bactDNA in CD patients regardless endoscopic evidences of mucosal lesions suggests first, the relative ease for bacterial fragments to cross the gut barrier. In fact, bacterial translocation is considered as a physiologic, naturally occurring event in healthy conditions that may turn into "pathological" under increased bacterial pressure. Therefore, and secondly, results suggest the utmost relevance of the immune system to counteract these translocation episodes. In this regard, the NOD2 genotype becomes relevant and, as shown in Figure 2D, a variant NOD2 genotype is associated with an exacerbated TNF-alpha response against bactDNA.

The results in the inflammatory soluble mediators are particularly relevant in the subgroup of patients on anti-TNF therapy, which are reflected in an increased drug consumption (Figure 2E) and an increased rate of intensified anti-TNF therapy in patients with bactDNA and a variant NOD2 genotype (Figure 2F). These findings are in line with previous reported results on the effect of variant NOD2 genotypes and bactDNA translocation on the efficacy of anti-TNF therapies [8]. The increased Th1 response, as a consequence of bactDNA translocation and an impaired *NOD2* function, might perpetuate the progression of the disease in this subgroup of patients, who are not adequately responding to the therapy schedule and might benefit from different strategies to prevent immunological derangements that may favor relapse. In fact, different studies have demonstrated that standard therapy schedules do not show the same efficacy in all CD patients, and efforts are needed to identify subgroups that may benefit from alternative, more aggressive early treatments to control and change the course of the disease [46-48].

Although we acknowledge the limitations derived from an observational study design and the reduced number of patients regarding the inflammatory response in anti-TNF therapy groups, the results obtained on the effect of bactDNA on the primary study end-point and clinical complications considered at 6 months offer solid evidence implicating bactDNA as a relevant factor inducing complications in CD.

In summary, the present study identifies bactDNA translocation as an independent risk factor of relapse in the short-term in CD patients in remission. The presence of this bacterial antigen is also independently associated with a significantly increased risk of other complications such as of hospitalization, switch of treatment or the startup of steroids. The increased inflammatory response to bactDNA translocation, particularly compromised in patients with a variant NOD2 genotype, is likely identifying a subgroup of CD patients susceptible of more aggressive early therapeutic approaches.

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Table 1. Patients' characteristics.

	All patients (n=288)	BactDNA-negative patients (n=190)	BactDNA-positive patients (n=98)	*P value*
Age (years)	42 ± 15	42 ± 15	43 ± 14	*0.61*
Weight (Kg)	70.65 ± 14.71	71.05 ± 12.80	69.88 ± 17.91	*0.16*
Gender (male/female), n (%)	153 (53.5%) / 134 (46.7%)	103 (54.2%) / 87 (45.8%)	50 (51.5%) / 48 (48.5%)	*0.76*
Smoking habit (yes / no / ex), n (%)	115 (40%) / 110 (38.2%) / 63 (21.8%)	72 (37.9%) / 80 (42.1%) / 38 (20%)	43 (43.8%) / 30 (30.6%) / 25 (25.5%)	*0.34*
Disease duration (months)	115,13 ± 114,49	114.55 ± 126.92	116.26 ± 85.66	*0.29*
Resection, n (%)	71 (25.3%)	51 (26.8%)	20 (20.4%)	*0.28*
CDAI	62.64 ± 33.14	62.08 ± 33.09	63.74 ± 33.45	*0.68*
Montreal A (age of onset), n (%)				
A1 (<=16)	16 (5.5%)	10 (5.3%)	6 (6.1%)	
A2 (17-40)	203 (70.5%)	136 (71.5%)	67 (68.4%)	*0.68*
A3 (>40)	69 (24%)	44 (23.2%)	25 (25.5%)	
Montreal L (location), n (%)				
L1	131 (45.4%)	91 (47.9%)	40 (40.8%)	
L2	65 (22.7%)	41 (21.5%)	24 (24.5%)	*0.3*
L3	79 (27.4%)	50 (26.4%)	29 (29.6%)	
L4	13 (4.5%)	8 (4.2%)	5 (5.1%)	
Montreal B (behavior), n (%)				
B1 (non-stricturing, non-penetrating)	138 (48%)	87 (45.8%)	51 (52%)	
B1p (non-stricturing, non-penetrating, penetrating perianal disease)	37 (12.8%)	24 (12.6%)	13 (13.2%)	
B2 (stricturing)	46 (16%)	32 (16.8%)	14 (14.3%)	*0.7*
B2p (stricturing, perianal disease associated)	13 (4.5%)	10 (5.3%)	3 (3.1%)	
B3 (penetrating)	42 (14.5%)	31 (16.3%)	11 (11.2%)	
B3p (penetrating, penetrating perianal disease)	12 (4.2%)	6 (3.2%)	6 (6.2%)	
Therapy, n (%)				
Mesalazine	65 (22.5%)	47 (24.7%)	18 (18.3%)	
Azathioprine	96 (34%)	64 (33.7%)	32 (32.6%)	
Metotrexate	9 (3.1%)	8 (4.2%)	1 (1%)	
Mesalazine and Azathioprine	16 (5.5%)	11 (5.7%)	5 (5.1%)	
Mesalazine and Steroids	4 (1.3%)	2 (1%)	2 (2%)	
Azathioprine + Steroids	4 (1.3%)	2 (1%)	2 (2%)	
Metotrexate + Steroids	1 (0.3%)	1 (0.5%)	-	
Infliximab	22 (7.6%)	15 (7.9%)	7 (7.1%)	*0.25*
Adalimumab	26 (9%)	12 (6.3%)	14 (14.3%)	
Infliximab + Azathioprine	15 (5.2%)	12 (6.3%)	3 (3.1%)	
Adalimumab + Azathioprine	9 (3.5%)	5 (2.6%)	5 (5.1%)	
Infliximab + Steroids	1 (0.3%)	1 (0.5%)	-	
Adalimumab + Steroids	4 (1.3%)	2 (1%)	2 (2%)	
Infliximab + Metotrexate	2 (0.7%)	-	2 (2%)	
Infliximab + Azathioprine + Steroids	1 (0.3%)	1 (0.5%)	-	
No therapy	12 (4.1%)	7 (3.6%)	5 (5.1%)	
Groups of therapy, n (%)				
No IS / no anti-TNF	78 (27.1%)	54 (28.4%)	24 (24.4%)	
IS	129 (44.7%)	88 (46.3%)	41 (41.8%)	*0.46*
Anti-TNF	50 (17.3%)	28 (14.7%)	22 (22.4%)	
IS + anti-TNF	31 (10.7%)	20 (10.5%)	11 (11.2%)	
CRP (mg/dL)	0.58 ± 1.11	0.59 ± 1.09	0.57 ± 1.17	*0.82*
Fecal Calprotectin (ug/g)	52.35 ± 35.73	54.95 ± 45.02	49.80 ± 23.65	*0.12*
Haemoglobin (g/dL)	13.93 ± 2.37	13.76 ± 1.49	14.26 ± 3.48	*0.22*
ESR (mm)	19.2 ± 15.8	18.8 ± 15.3	19.7 ± 15.5	*0.15*
Albumin (g/dL)	4.13 ± 4.62	4.11 ± 4.61	4.13 ± 4.51	*0.53*
Total WBCs (mm^3)	6880.5 ± 2648.2	6721.4 ± 2579.4	7186.2 ± 2765.2	*0.13*
Temperature (ºC)	36.07 ± 0.22	36.05 ± 0.17	36.12 ± 0.31	*0.18*
Pulse rate (bpm)	70.72 ± 6.21	70.30 ± 5.90	71.5 ± 6.75	*0.13*

All values shown as mean ± SD or percentage. P values correspond to he comparison between bactDNA-negative vs bactDNA-positive patients. CDAI: Crohn's Disease Activity Index; IS: immunosuppressors; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; WBC: white blood cells.

Table 2. Bacterial DNA species identification in CD patients.

Bacterial DNA identification	Patients n (% of bactDNA+ patients)	Concentration of amplified bactDNA fragments (ng/uL)	Family / (gram staining)	Patients n (% of bactDNA+ patients)
Escherichia coli	38 (38.77%)	28.2 ± 12.3	Enterobacteriaceae (gram-negative)	76 (77.6%)
Klebsiella pneumoniae	13 (13.26%)	26.5 ± 10.8		
Klebsiella oxytoca	1 (1.02%)	16.5		
Bacteroides intestinalis	5 (5.10%)	30.6 ± 15.1		
Bacteroides uniformis	2 (2.04%)	21.8 ± 6.1		
Bacteroides fragilis	1 (1.02%)	25.1		
Bacteroides faecis	1 (1.02%)	30.6		
Proteus vulgaris	2 (2.04%)	18.4 ± 8.4		
Proteus mirabilis	1 (1.02%)	20.6		
Proteus morganii	1 (1.02%)	20.8		
Enterobacter cloacae	3 (3.06%)	33.4 ± 9.8		
Enterobacter aerogenes	1 (1.02%)	22.8		
Shigella flexneri	2 (2.04%)	27.1 ± 7.2		
Shigella sonnei	1 (1.02%)	23.2		
Citrobacter freundi	2 (2.04%)	22.7 ± 9.4		
Morganella morganii	1 (1.02%)	28.6		
Serratia marcescens	1 (1.02%)	31.4		
Staphylococcus aureus	8 (8.16%)	31.4 ± 8.5	Staphylococcaceae (gram-positive)	8 (8.2%)
Enterococcus faecalis	7 (7.14%)	22.4 ± 10.5	Enterococcaceae (gram-positive)	7 (7.1%)
Streptococcus pneumoniae	3 (3.06%)	27.4 ± 6.6	Streptococcaceae (gram-positive)	7 (7.1%)
Streptococcus pyogenes	3 (3.06%)	32.4 ± 10.4		
Streptococcus agalactiae	1 (1.02%)	26.2		

Table 3. Univariate and multivariate survival analyses of relapse at 6 months.

Univariate Survival Analysis at 6 months

		Patients who relapse n (%)	*P value* (chi square)	Mean of survival (95% CI)	Log Rank test *P value*
Bacterial DNA	yes	32 / 98 (32.6%)	0.001	5.24 (4.92 - 5.55)	<0.001
	no	8 / 190 (4.2%)		5.94 (5.88 - 6.00)	
Group of therapy	no IS / no anti-TNF	5 / 78 (6.4%)	0.004	5.86 (5.70 - 6.02)	
	IS	16 / 129 (12.4%)		5.66 (5.46 - 5.86)	0.165
	anti-TNF	9 / 50 (18%)		5.78 (5.57 - 5.99)	0.045
	IS + anti-TNF	10 / 30 (33.3%)		5.33 (4.75 - 5.91)	<0.001
NOD2 genotype	wild type	24 / 174 (13.8%)	0.954	5.69 (5.53 - 5.84)	0.995
	variant	16 / 114 (14%)		5.72 (5.52 - 5.92)	

Multivariate Survival Analysis at 6 months

	Hazard ratio (95% CI)	*P value*
Bacterial DNA (yes / no)	8.75 (4.02 - 19.06)	<0.001
Group of therapy		
IS (vs no IS / no anti-TNF)	2.06 (0.75 - 5.61)	0.160
anti-TNF (vs no IS / no anti-TNF)	2.16 (0.72 - 6.45)	0.169
IS + anti-TNF (vs no IS / no anti-TNF)	5.43 (1.86 - 15.89)	0.002

IS: immunosuppressor; CI: confidence interval

Table 4. Clinical complications and analytical parameters at 6 months.

A) Patients distributed by gut bacterial DNA translocation in blood

Events/Analytical parameters at 6 motnhs		BactDNA-negative at baseline (n=190)	BactDNA-positive at baseline (n=98)	*P value*
Number of flares	1	5	20	0.549
	2	1	8	
Hospitalization, n (%)		3 (1.6%)	16 (16.3%)	<0.001
Surgery, n (%)		0	5 (5.1%)	0.003
Switch of treatment, n (%)		14 (7.4%)	20 (20.4%)	0.005
Anti-TNF startup, n (%)		1 (0.5%)	6 (6.1%)	0.018
Anti-TNF escalation, n (%)		3 (1.6%)	9 (9.2%)	0.008
Anti-TNF switch, n (%)		3 (1.6%)	1 (1.1%)	0.830
Steroids startup, n (%)		4 (2.1%)	16 (16.3%)	<0.001
Azathioprine startup, n (%)		8 (4.2%)	1 (1.1%)	0.302
CRP (mg/dL)		0.75 ± 2.01	0.87 ± 1.52	0.623
Calprotectin (ug/g)		60.06 ± 98.5	151.35 ± 188.98	0.001
Haemoglobin (g/dL)		13.73 ± 1.45	13.86 ± 1.47	0.457
ESR 6 (mm)		16.47 ± 13.56	15.94 ± 12.08	0.780
Albumin (g/dL)		4.08 ± 0.54	4.06 ± 0.45	0.868
Total WBCs (mm^3)		6952.45 ± 3607.85	8411.45 ± 11132.86	0.102

CRP: C-reactive protein; ESR: erytrocyte sedimentation rate; WBC: white blood cells.

B) Patients distributed by group of therapy

Events/Analytical parameters at 6 motnhs		no IS / no anti-TNF at baseline (n=78)	IS at baseline (n=129)	anti-TNF at baseline (n=50)	IS + anti-TNF at baseline (n=31)	*P value*
Number of flares	1	2	9	6	8	0.549
	2	2	4	2	1	
Hospitalization, n (%)		2 (2.6%)	9 (7%)	5 (10%)	3 (9.7%)	<0.001
Surgery, n (%)		2 (2.6%)	1 (0.7%)	1 (2%)	1 (3.2%)	0.399
Switch of treatment, n (%)		6 (7.7%)	16 (12.4%)	6 (12%)	6 (19.4%)	0.005
Anti-TNF startup, n (%)		1 (1.3%)	3 (2.3%)	-	-	0.180
Anti-TNF escalation, n (%)		2 (2.6%)	3 (2.3%)	5 (10%)	2 (6.6%)	0.008
Anti-TNF switch, n (%)		1 (1.3%)	2 (1.6%)	1 (2%)	-	0.830
Steroids startup, n (%)		1 (1.3%)	12 (9.3%)	4 (8%)	3 (9.7%)	<0.001
Azathioprine startup, n (%)		5 (6.4%)	1 (0.7%)	-	-	0.102
CRP (mg/dL)		0.68 ± 1,44	0.67 ± 1.47	1.23 ± 3.02	0.89 ± 1.55	0.309
Calprotectin (ug/g)		61.78 ± 77.24	76.29 ± 124.24	116.35 ± 183.43	149.47 ± 183.26	0.026
Haemoglobin (g/dL)		13.69 ± 1.60	13.89 ± 1.35	13.72 ± 1.37	13.59 ± 1.68	0.637
ESR (mm)		15.38 ± 13.60	15.20 ± 11.23	17.42 ± 14.96	21.24 ± 15.21	0.190
Albumin (g/dL)		4.08 ± 0.40	4.14 ± 0.55	4.07 ± 0.55	3.84 ± 0.44	0.071
Total WBCs (mm^3)		8473.51 ± 12356.47	6460.83 ± 3735.95	7989.28 ± 2782.07	8195.17 ± 4184.48	0.198

CRP: C-reactive protein; ESR: erytrocyte sedimentation rate; WBC: white blood cells.

Table 5. Distribution of study variables and complications at 6 months in patients with colonoscopy at admission.

		No lessions at baseline (n=50)	Aphtous lessions at baseline (n=40)	Ulcers at baseline (n=13)	*P value*
BactDNA, n (%)	(no / yes)	32 (64%) / 18 (36%)	28 (70%) / 12 (30%)	10 (77%) / 3 (23%)	0.632
NOD2 genotype, n (%)	(wt / variant)	22 (44%) / 28 (66%)	13 (32.5%) / 27 (67.5%)	3 (23%) / 7 (77%)	0.289
Group of therapy, n (%)					
	No IS / no anti-TNF	11 (22%)	12 (30%)	4 (30.7%)	
	IS	23 (46%)	15 (37.5%)	6 (46.2%)	0.565
	anti-TNF	7 (14%)	10 (25%)	2 (15.4%)	
	IS + anti-TNF	9 (18%)	3 (7.5%)	1 (7.7%)	
Calprotectin (ug/g)		31.86 ± 16.58	55.06 ± 28.90	42.75 ± 24.83	0.341
Relapse at 6 months	(no / yes)	44 (88%) / 6 (12%)	33 (82.5%) / 7 (17.5%)	10 (77%) / 3 (23%)	0.561
Hospitalization at 6 months	(no / yes)	47 (94%) / 3 (6%)	37 (92.5%) / 3 (7.5%)	12 (92.3%) / 1 (7.7%)	0.952
Surgery at 6 months	(no / yes)	50 (100%) / 0	38 (95%) / 2 (5%)	13 (100%) / 0	0.201
Switch of treatment at 6 months	(no / yes)	46 (92%) / 4 (8%)	32 (80%) / 8 (20%)	11 (84.6%) / 2 (15.4%)	0.593
Calprotectin at 6 months (ug/g)		97.60 ± 165.68	86.32 ± 120-04	107.50 ± 167.78	0.524

bactDNA: bacterial DNA; wt: wild type; IS: immunosuppressor

Figure legends.

Figure 1. Overall survival Kaplan-Meier curves at 6 months in CD patients according to baseline bactDNA translocation (A) and group of therapy (B). BactDNA: bacterial DNA; IS: immunosuppressor.

Figure 2. Soluble inflammatory response in CD patients in remission. A) Serum TNF-alpha levels in CD patients distributed by the presence of bactDNA translocation in blood. B) Serum TNF-alpha levels in CD patients distributed by the *NOD2* genotype. C) Serum TNF-alpha levels in CD patients distributed by the group of therapy. D) Serum TNF-alpha levels in CD patients on anti-TNF therapy distributed by the combination of bactDNA translocation and *NOD2* genotype. E) Serum free anti-TNF-alpha levels in CD patients on anti-TNF therapy distributed by the combination of bactDNA translocation and *NOD2* genotype. F) Percentage of intensifeid CD patients on anti-TNF therapy distributed by the combination of bactDNA translocation and *NOD2* genotype. * $p<0.05$ compared with bactDNA-negative, wildtype *NOD2* or the combination of both; $^{\$}$ $p<0.05$ compared with the rest of groups. BactDNA: bacterial DNA; IS: immunosuppressor.